Isolation of pathogenic *Yersinia enterocolitica* from foods

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ABSTRACT

Many selective enrichment methods for the isolation of *Yersinia enterocolitica* from foods have been described. However, no single isolation procedure was available for the recovery and identification of different plasmid-bearing pathogenic serotypes. Two improved procedures based on homogenization and swabbing techniques are discussed for selective enrichment, isolation, identification, and maintenance of a wide spectrum of plasmid-bearing virulent serotypes of *Y. enterocolitica* from different food sources. The plasmid-bearing pathogenic serotypes were identified and isolated using Congo red binding and low-calcium-response tests. Further confirmation by multiplex polymerase chain reaction employed primers directed at the chromosomal *ail* and plasmid-borne *virF* genes which are present only in pathogenic strains. The methods were applied to pig slaughter house samples and were effective in isolating plasmid-bearing virulent strains of *Y. enterocolitica* from naturally contaminated porcine tongues. Strains isolated from food samples and tongue expressed plasmid-associated phenotypes and mouse pathogenicity.

INTRODUCTION

Yersinia enterocolitica is a short rod-shaped gram-negative, facultatively cold-tolerant anaerobic bacterium belonging to the genus *Yersinia* in the Enterobacteriaceae family. The organism is recognized as a foodborne pathogen and a large number of food-associated outbreaks of yersiniosis have been reported. In developed countries, *Y. enterocolitica* can be isolated from 1-2% of all human cases of acute enteritis (1).

Common food vehicles in outbreaks of yersiniosis are meat (particularly pork), milk, dairy products, powdered milk, cheese, tofu and raw vegetables (1-4). Since yersiniae can grow at low temperatures, even refrigerated foods are potential vehicles for the growth of these organisms (1-3). The majority of these food isolates differ in biochemical and serological characteristics from typical clinical strains and are usually called 'non-pathogenic' or 'environmental' *Yersinia* strains. The strains of all serotypes implicated in human disease harbor a virulence-associated plasmid of 70 to 75 kb pairs (1-3).

The increasing incidence of *Y. enterocolitica* infections and the role of foods in some outbreaks of yersiniosis has led to the development of a wide variety of methods for the isolation of *Y. enterocolitica* from foods (3, 5). The unstable nature of the virulence plasmid (1-3, 6, 7) complicates the isolation of plasmid-bearing virulent *Y. enterocolitica* (YEP^+) by causing the overgrowth of virulent cells by plasmid less revertants eventually leading to a completely avirulent culture. Since the population of *Y. enterocolitica* in foods is usually low and natural microflora tend to suppress the growth of this organism (8), isolation methods usually involve enrichment followed by plating onto selective media. The purpose of this chapter to review the research on the development of a single procedure for selective enrichment, isolation, identification and maintenance of pathogenic YEP^+ serotypes from food samples harboring this organism by homogenization and swabbing techniques.

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Historical Aspects of Enrichment, Plating, and Identification

Enrichment

Traditional methods employ the use of prolonged enrichment at refrigeration temperatures to take advantage of the psychrotrophic nature of *Y. enterocolitica* and to suppress the growth of background flora. At this low temperature, the growth rate of competitive bacteria is decreased sufficiently to enable *Y. enetrocolitica* to grow to number necessary for isolation on plating media. Media used for this 'cold enrichment' include buffers like phosphate-buffered saline (PBS), PBS modified by addition of 1% sorbitol and 0.15% bile salts (PBSSB), PBS with 0.5% peptone, PBS supplemented with 1% mannitol, PBS with 5% peptone, PBS with peptone and cyclohexamide, tryptone soy broth, trypticase soy broth or tris-buffered peptone water, pH 8.0 (5, 9). Due to the extended time period required to perform this type of method, efforts have been made to devise selective enrichment techniques employing shorter incubation times and higher temperature, thus, making them more practical for routine use (1, 3, 5, 9).

Several other enrichment procedures involving incubation at higher temperatures for a shorter period and using selective media have been proposed. However, high levels of indigenous microorganisms can overgrow and mask the presence of *Y. enterocolitica* strains (1, 3, 5, 9) including non-pathogenic *Yersinia* strains which are frequently isolated from food samples and are atypical in some biochemical reactions (3, 9). It also appears that the efficiency of *Y. enterocolitica* enrichment techniques varies greatly with serotype and depends on the type of food being tested. Enrichment media containing the selective agent Irgasan, applicable for a wide spectrum of *Y. enterocolitica* strains, have been described but are only effective for recovery of the organism from meat samples and require two weeks of enrichment (4). No single enrichment procedure has been shown to recover a broad spectrum of pathogenic YEP$^+$ serotypes from a variety of foods.

Plating media

Since there is no specific medium for the isolation of YEP$^+$ strains, different agar plating media have been used to isolate presumptive *Y. enterocolitica* from foods. Initially agar media like MacConkey agar (MAC), Salmonella-Shigella agar, desoxycholate citrate agar or bismuth sulphite agar which were designed for the isolation of enteropathogens were used for *Y. enterocolitica*. Schiemann (10) developed cefsulodin-irgasan-novobiocin (CIN) agar, as a selective and differential agar medium for *Y. enterocolitica*. Yersiniae produce red colored 'bullseye' colonies on CIN agar. CIN agar was found to be inhibitory to most of the gram-negative bacteria but some gram-negative bacteria show a colony appearance similar to *Yersinia* (5, 9). Moreover, on CIN agar, colonies of pathogenic and environmental *Yersinia* are similar in appearance (11).

Identification

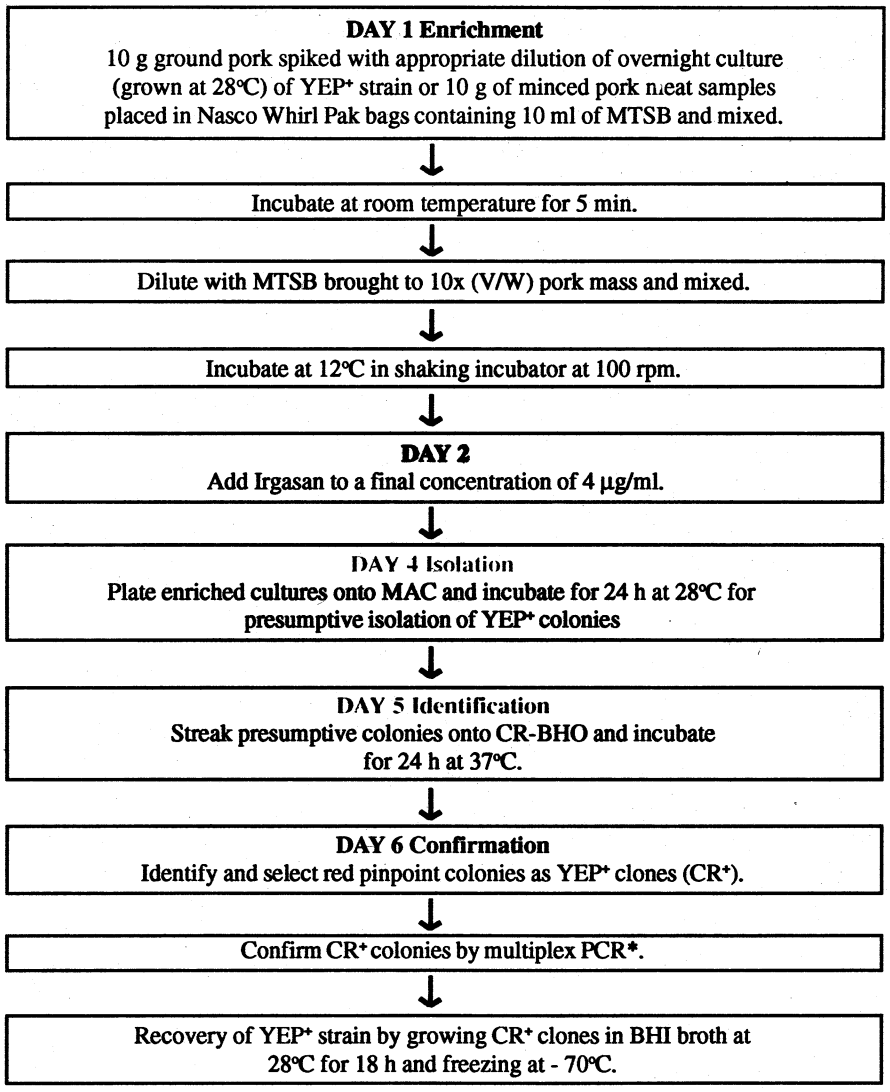
Since colonies of *Y. enterocolitica* are presumptive on the plating media, these isolates should be identified as YEP$^+$ strains. Biochemical reactions, serotyping, biotyping, and virulence testing are essential for differentiation between YEP$^+$, plasmid less avirulent *Y. enterocolitica* (YEP$^-$), environmental *Yersinia* strains and other *Yersinia*-like presumptive organisms. Biochemical tests using systems such as API 20E give similar reactions among these organisms and are not conclusive. Serotyping involving major O and H factors differentiate between pathogenic and environmental *Y. enterocolitica* but failed to discriminate between YEP$^+$ and YEP$^-$ strains. Biotyping involves biochemical tests which do not show the presence of the virulence plasmid. Thus, it does not identify YEP$^+$ strains. Several *in vitro* tests including colony morphology, autoagglutination (AA), serum resistance, tissue culture detachment, hydrophobicity(HP), low-calcium response (Lcr), crystal violet (CV) binding, Congo red (CR) binding, isolation of the virulence plasmid and colony hybridization techniques have been described to determine the potential virulence of *Yersinia* isolates (6, 12). Unfortunately, methods described in the literature do not include tests for confirmation of virulence of presumptive or known *Y. enterocolitica* isolates recovered from selective agars as an integral part of the detection method. The most rapid procedure available for the isolation of a wide spectrum of *Y. enterocolitica* strains does not include the identification of isolates as plasmid-bearing virulent strains (4).

Development of a single procedure for selective enrichment, isolation, identification and maintenance of pathogenic YEP$^+$ serotypes from pork samples

Pigs and pork meat have been identified as

Selective Enrichment, Isolation, and Identification of Plasmid-Bearing Virulent Strains of *Yersinia enterocolitica* from Pork Samples

FLOW CHART 1



*Confirmation of CR+ clones as YEP+ strain by multiplex PCR using chromosomal *ail* gene and *vir*F gene from virulence plasmid.

important reservoirs of *Yersinia enterocolitica*, including those species and serotypes which have been associated with human illness (3). Therefore, research on the development of a method for the recovery of a wide variety of serotypes of YEP$^+$ strains from pork samples was initiated. The ability of *Y. enterocolitica* to grow in the presence of selective agents Irgasan, ticarcillin, and potassium chlorate (13) led Bhaduri et al. (14) to test the suitability of use these agents in enrichment procedures. Irgasan (Ciba-Geigy Corp., Greensboro, NC) was found to be more suitable in allowing the growth of *Y. enterocolitica*. Enrichment methods for *Y. enterocolitica* reported in the literature permit recovery, but they do not specifically confirm the presence of YEP$^+$ strains. Bhaduri et al. (14) developed a single procedure using enrichment in modified trypticase soy broth (MTSB) (Difco Laboratories, Detroit, Michigan) containing 0.2% bile salts no. 3 (Difco) at 12°C for isolation, identification and maintenance of YEP$^+$ strains from pork samples (see FLOW CHART 1). Ground pork artificially contaminated with varying concentrations of YEP$^+$ strains of five serotypes including GER (O:3), EWMS (O:13), PT18-1 (O:5, O:27), O:TAC (O:TACOMA) and WA (O:8) was tested by this enrichment method. The addition of Irgasan plays a critical role in the enrichment of YEP$^+$ strains. Since the presence of Irgasan suppresses the growth of pure YEP$^+$ cultures grown in MTSB when added at the onset of growth and does not when added after the lag phase, it was not added initially in the enrichment medium. In tests to recover YEP$^+$ strains from ground pork, Irgasan was added at 24 h, 48 h, and 72 h. It was found that addition of Irgasan at 24 h gave the best recovery of YEP$^+$ serotypes (14). This timing may reduce the inhibitory effect of the antibiotic (13). The addition of

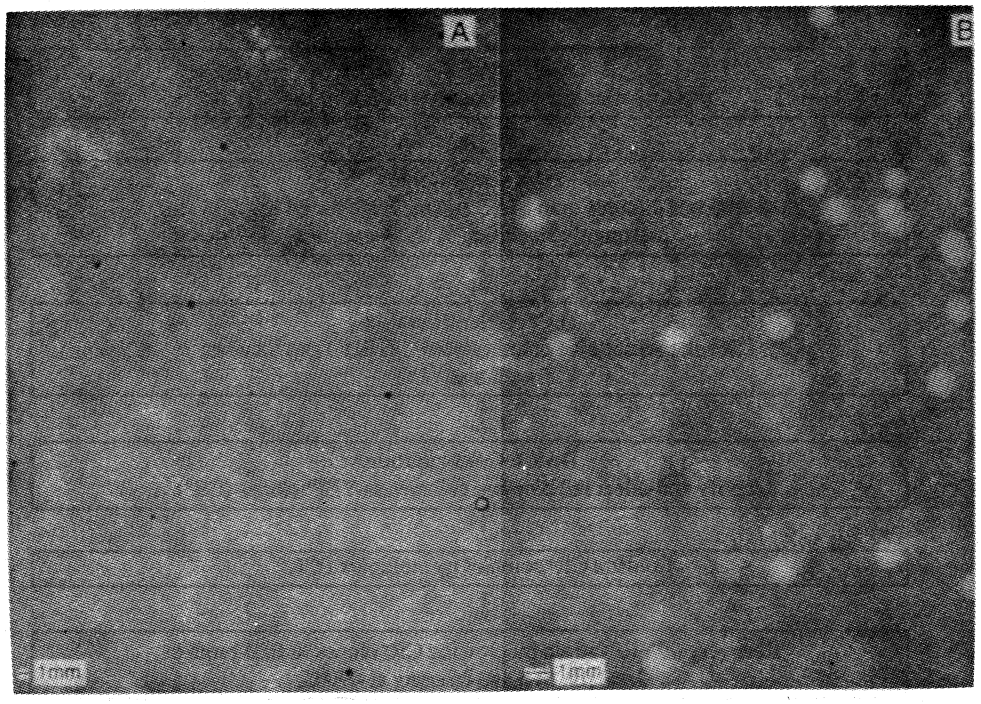


FIG. 1. Identification of virulent plasmid-bearing clones of YEP$^+$ from pork meat samples by CR Binding Technique. (A) Red pinpoint colonies (0.36 mm in original diameter) showing CR binding and Lcr were identified as virulent YEP$^+$ strain. (B) Avirulent YEP$^-$ strain used as a negative control showing large white colonies (1.37 mm in original diameter). (14, 15).

Irgasan after 24 h (day 2) and incubation for an additional 48 h (day 4) at 12°C allowed *Yersinia* strains to grow to detectable levels even in the presence of competing microflora. It was also determined that sampling should be done at 48 h of incubation to allow any *Yersinia* present time to reach detectable levels and to avoid sampling after competing microflora begin to predominate. This technique enhances isolation of YEP$^+$ strains in the presence of competing microflora through the selection of incubation temperature, sampling schedule, and timing of antibiotic addition.

Both MAC and CIN agars were used for presumptive isolation of YEP$^+$ serotypes. Presumptive colonies were first identified as YEP$^+$ strains by CR binding and Lcr techniques. Pathogenic YEP$^+$ strains appeared as red pinpoint colonies (CR$^+$) on low calcium (238 μM) CR (Sigma Chemical Co., St. Louis, Mo) brain heart infusion (Difco) agarose (Gibco BRL, Gaithersburg, Md) supplemented with 0.1% magnesium chloride (CR-BHO) at 37°C for 18 to 24 h incubation (Fig. 1A). The colony pigmentation was due to CR binding and their pinpoint size was due to Lcr (15). The identification of YEP$^+$ strains on CR-BHO allows the recovery of YEP$^+$ colonies harboring the virulence plasmid (14, 15). The presumptive colonies isolated from MAC agar were identified as YEP$^+$ clones. CIN agar did not prove to be effective in isolating YEP$^+$ clones since all typical presumptive colonies tested negative. The failure of CIN agar to isolate YEP$^+$ presumptives was likely due to the similar colonial morphology of other organisms present in the samples (11). Moreover, CIN agar may inhibit the growth of YEP$^+$ strains due its antibiotic content. MAC agar gave consistently higher numbers of presumptives which

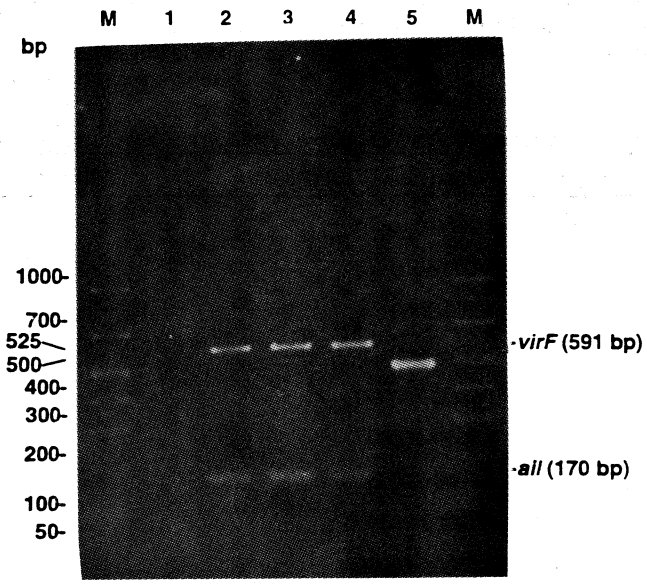


FIG. 2. Confirmation of CR$^+$ clones isolated from artificially contaminated ground pork as YEP$^+$ strain by multiplex PCR using chromosomal *ail* Gene and *virF* gene from virulence plasmid. Lanes; M, 50 to 1000 bp ladder marker; 1, negative control with no template; 2 and 3 CR$^+$ colony showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 4, positive control with purified chromosomal DNA from YEP$^+$ strain showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 5, positive control for PCR assay with λ as DNA template. (14)

were eventually shown to be YEP$^+$ strains. Enrichment and isolation of presumptive YEP$^+$ strains were followed with identification by a PCR assay and testing of plasmid-associated virulence phenotypes. The methods reported in the literature can potentially recover *Yersinia,* but do not attempt to specifically confirm the presence of virulent strains after enrichment. The CR$^+$ clones from MAC agar are confirmed by multiplex PCR using chromosomal *ail* gene and *virF* gene from the virulence plasmid (16). Each primer pair for the *ail* and *virF* genes amplified a 170 bp product from the chromosome and a 591 bp product from the virulence plasmid (Fig. 2, lanes 2 and 3 respectively). The presence of the *ail* gene specifically differentiates YEP$^+$ strains from environmental *Y. enterocolitica* and *Y. pseudotuberculosis* lacking this gene (16, 17). The presence of the *virF* gene demonstrates the presence of the virulence plasmid which confers the plasmid-associated phenotypes. Thus, the YEP$^+$ virulent strains were identified by both virulence plasmid-associated phenotypic expression and the presence of specific virulence genes.

It takes 6 days to complete sample enrichment through confirmation of YEP$^+$ strains. As low as 9 CFU of YEP$^+$ per gm of spiked ground pork can be recovered (Table 1). This technique has been successfully applied in the recovery of different YEP$^+$ strains of five serotypes including GER (O:3), EWMS (O:13), PT18-1 (O:5, O:27), O:TAC (O:TACOMA) and WA (O:8) from artificially contaminated ground pork. The procedure was tested on unspiked pork meat samples including ground pork (twenty samples), head meat (ten samples) and tongue (thirty samples). Only tongue was found to contain YEP$^+$ strains (Table 2). The standard cold enrichment technique at 4°C for 4 weeks failed to isolate YEP$^+$ strains from any of these pork meat samples with MAC and CIN agar were used as selective media. The successful isolation of YEP$^+$ strains from naturally contaminated tongue verified the effectiveness of this method. Eleven of 30 tongues analysed (~37%) were positive by both CR binding, Lcr and PCR analysis showing a 170 bp product from the chromosome and a 591 bp product from the virulence plasmid (Fig. 3 lanes 2-8). All isolates from tongues were serotype O:3 and designated as SB serotype O:3.

TABLE 1. Sensitivity of recovery of YEP$^+$ strain from artificially contaminated ground pork

YEP$^+$ strain CFU/gm	Initial ratio (YEP$^+$:BKG[a])	YEP$^+$ strain confirmation[b]
25000	1:40-400	+
9371	1:100-1000	+
5160	1:200-2000	+
3000	1:300-3000	+
518	1:2000-20000	+
300	1:3000-30000	+
195	1:5000-50000	+
96	1:10000-100000	+
52	1:20000-200000	+
30	1:30000-300000	+
9	1:100000-1000000	+
5	1:200000-2000000	-
3	1:300000-3000000	-

[a]BKG: Background microflora.
[b]Confirmed by Lcr-CR binding (YEP$^+$ cells appeared as red pinpoint colonies on CR-BHO) and PCR on day 6. +, detected; -not detected. (14)

TABLE 2. Recovery and confirmation of YEP$^+$ strains from pork samples (14)

Type of pork sample	Number of samples tested	Samples having virulent YEP$^+$ strains
Ground Meat	20	0
Head Meat	10	0
Tongue	30	11

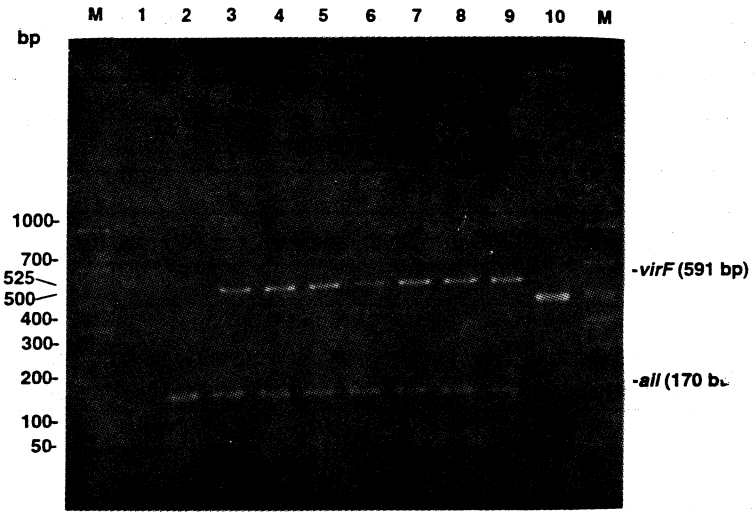


FIG. 3. Confirmation of CR$^+$ clones isolated from naturally contaminated porcine tongue as YEP$^+$ strains by multiplex PCR using chromosomal *ail* gene and *virF* gene from virulence plasmid. Lanes; M, 50 to 1000 bp ladder marker; 1, negative control with no template; 2-8, CR$^+$ colonies showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 9, positive control with purified chromosomal DNA from YEP$^+$ strain showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 10, positive control for PCR assay with λ as DNA template. (14)

Recent reports also indicate the emergence of serotype O:3 of *Y. enterocolitica* as the major cause gastroenteritis in the United States (18-20).

YEP$^+$ strains isolated from both artificially contaminated ground pork and naturally contaminated tongues were found to express plasmid-associated virulence characteristics (6, 12) including colonial morphology (appearance of small colony size of 1.13 mm), CV binding (appearance of dark violet colony), Lcr in low calcium medium (appearance of pinpoint colony size of 0.36 mm), CR uptake (appearance of red pinpoint colony size of 0.36 mm), HP by latex particle agglutination (LPA) and AA. The mouse virulence test (21) of YEP$^+$ strains isolated from spiked ground pork and naturally contaminated tongue was positive. These results showed that the organism retained the virulence plasmid-associated phenotypes and pathogenicity after isolation from food by this procedure.

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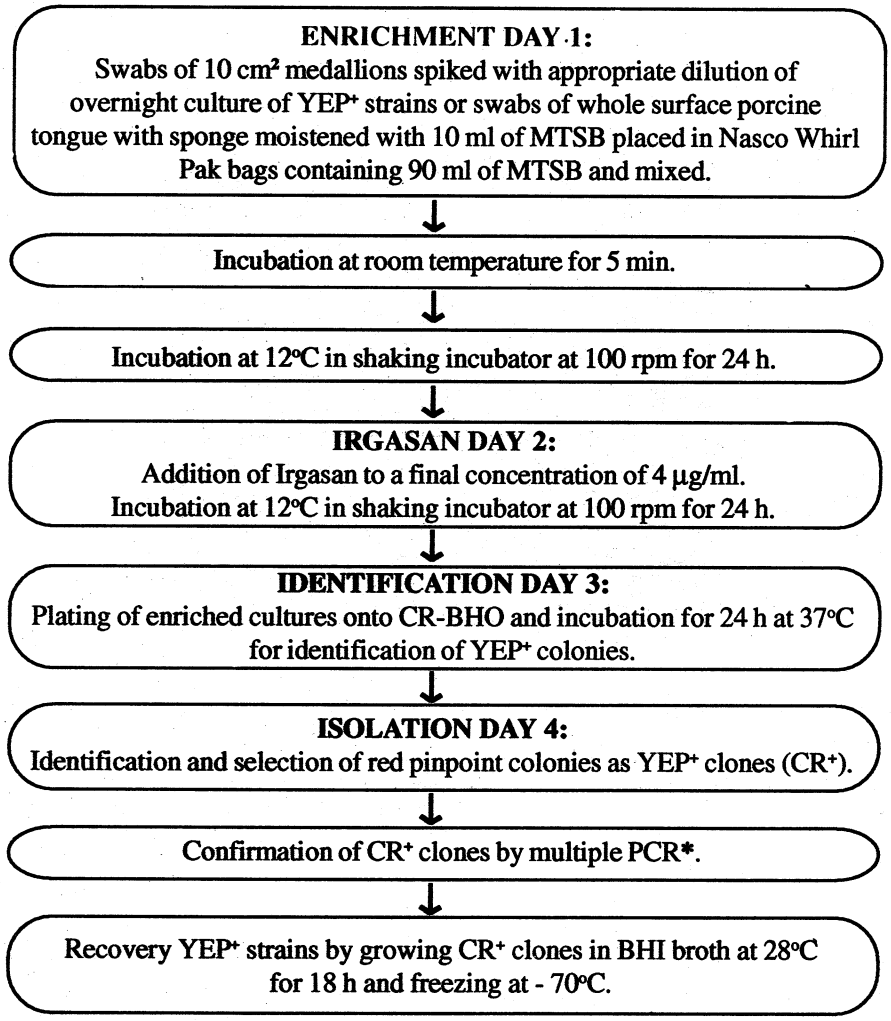
Direct Detection and Isolation of Plasmid-bearing Virulent Clones of *Yersinia enterocolitica* from Various Foods

FLOW CHART 2



*Confirmation of CR+ clones as YEP+ strain by multiplex PCR using chromosomal *ail* gene and *vir*F gene from virulence plasmid.

MTSB: Modified trypticase soy broth.

CR-BHO: Low-calcium (238 µM) Congo red brain heart infusion agarose.

Development of direct detection, isolation, and maintenance of pathogenic YEP+ serotypes from various food samples

Despite the advantages of the above method, there were several areas where additional improvements could be realized. Food matrices can inhibit the enrichment of YEP+ strains. The initial isolation of presumptive *Y. enterocolitica* from enriched samples on MacConkey agar adds an extra plating step and the picking of presumptive *Y. enterocolitica* requires skilled recognition and handling of the colonies (14). The unstable nature of the virulence plasmid (1-3, 6, 7, 15) complicates the detection of YEP+ strains, since isolation steps may lead to plasmid loss and the associated phenotypic characteristics for colony differentiation. The handling and preparation of food samples is time consuming. Finally, there was a need for development of a single medium enrichment and isolation procedure for pathogenic YEP+ serotypes from a variety of foods.

Food surfaces are often the primary site of bacterial contamination. Non-destructive swabbing has been used widely as a surface sampling procedure for the isolation and detection of a wide variety of food borne pathogens from meat (22). No single procedure has been described in the literature for simultaneous detection and isolation of various YEP+ serotypes from a variety of foods. For these reasons, it was decided to develop a technique to directly detect and isolate YEP+ serotypes by enriching swabs of artificially contaminated pork chops, ground pork, cheese, and zucchini in a single enrichment medium and applying CR binding, Lcr and PCR for confirmation (23). Medallions of each type of food were prepared using a 10 cm^2 coring tool. Medallions were artificially surface contaminated with varying concentrations of YEP+ strains of five serotypes including GER (O:3), EWMS (O:13), PT18-1 (O:5, O:27), O:TAC (O:TACOMA) and WA (O:8) and sampled by swabbing the surface with a sterile 5 × 5 × 1.25 cm cellulose sponge moistened with MTSB containing 0.2% bile salts. Swabs from each food sample were enriched in a single medium of MTSB containing 0.2% bile salts at 12°C for direct detection and isolation (see FLOW CHART 2). Enrichment of swabs was optimized using pork chops, ground pork, cheese, and zucchini artificially contaminated with various concentrations of YEP+ strain GER (serotype O: 3) (Table 3). The addition of Irgasan at 24 h (day 2) gave the best recovery of YEP+ strains as mentioned previously (14). Since the actual food sample was not used, there was a lower level of competing microflora, which allowed better growth of YEP+ strains during enrichment and enhanced recovery. The YEP+ strains could be detected and isolated when directly plated on CR-BHO (15) after 48 h of total incubation at 12°C (day 3). The YEP+ colonies from artificially contaminated pork chops (Fig. 4A), ground pork (Fig. 4B), cheese (Fig. 4C), and zucchini (Fig. 4D) all appeared as CR+ (red pin point) colonies (day 4). Thus, YEP+ strains from each food sample were identified as harboring the virulence plasmid. This modified protocol reduced the original method of Bhaduri et al. (14) by 2 days by eliminating 1 day of enrichment and 1 day for plating on MAC agar. The CR+ clones were further confirmed as YEP+ strains by multiplex PCR using primers directed to the *ail* and *virF* genes amplifying a 170-bp product from the chromosome and 591-bp product from the virulence plasmid (16), respectively (Fig. 5 pork chops lanes 2-4,

TABLE 3. Sensitivity of Recovery of YEP+ GER strain (serotype O:3) from artificially contaminated foods

Concentration of YEP+ strain (CFU/cm^2)	Recovery of YEP+ strain[a]			
	Pork Chop	Ground Pork	Cheese	Zucchini
10	+	+	+	+
1	+	+	+	+
0.5	+	+	+	+
0.1	-	-	-	-

[a]Detected and isolated by Lcr-CR binding (YEP+cells as appeared as red pin point colonies on CR-BHO) and confirmed by PCR on day 4 (pork chops and ground pork) and day 5 (cheese and zucchini) for 0.5 CFU/cm^2 concentration, respectively. + detected; - not detected. (23)

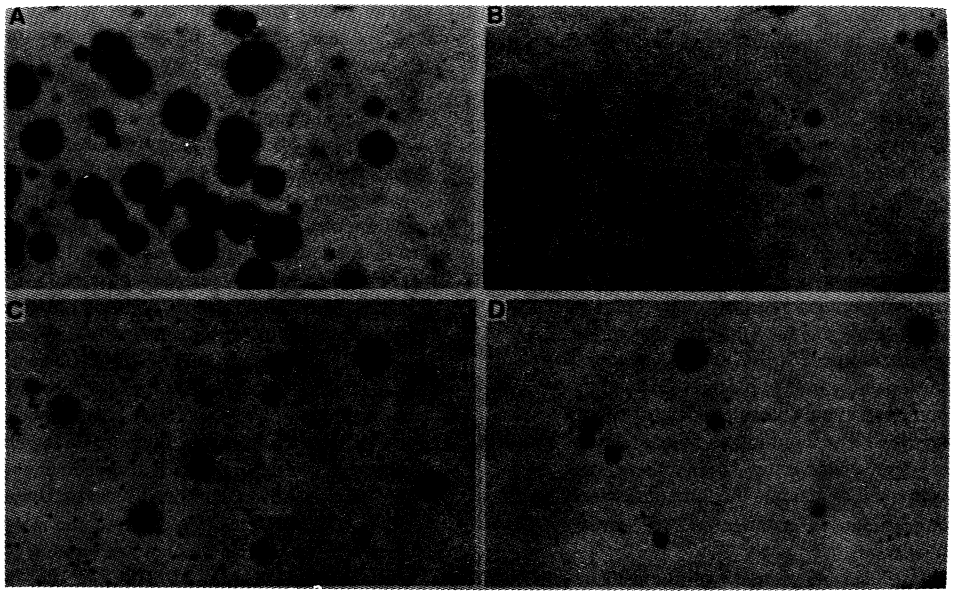


Fig. 4. Direct detection and isolation of YEP$^+$ strains as red pin point colonies on CR-BHO from artificially contaminated pork chops (A), ground pork (B), cheese (C), and zucchini (D). (23)

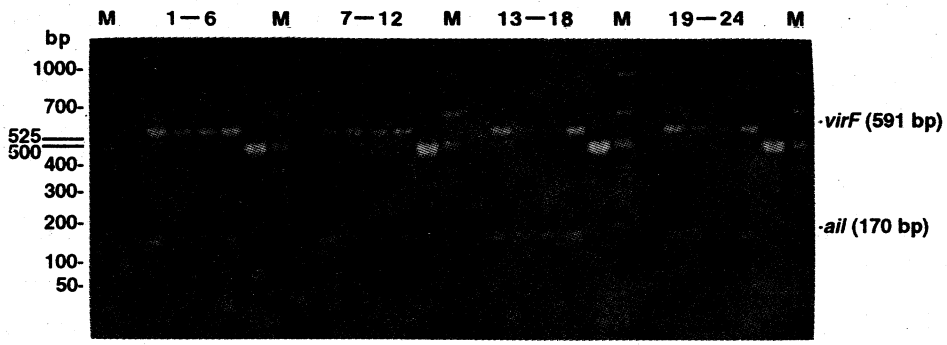


Fig 5. Confirmation of CR$^+$ clones isolated from artificially contaminated various foods as YEP$^+$ strain by multiplex PCR using chromosomal *ail* Gene and *virF* gene from virulence plasmid. Lanes; M, 50 to 1000 bp ladder marker; Negative control with no template: 1,7,13, & 19; CR$^+$ colony showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively: Pork chops 2-4; Ground pork 8-10; Cheese 14-16; Zucchini 20-22; Positive control with purified DNA from YEP$^+$ strain showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively: 5, 11,17, & 23; Positive control for PCR assay with λ as DNA template: 6, 12, 18, & 24. (23)

ground pork lanes 8-10, cheese 14-16, and zucchini lanes 20-22). Thus, the YEP$^+$ virulent strains were identified by both virulence plasmid-associated phenotypic expression and the presence of specific virulence genes.

This method can be completed in 4 days from sample enrichment to isolation including confirmation by multiplex PCR, and can recover YEP$^+$ strains from

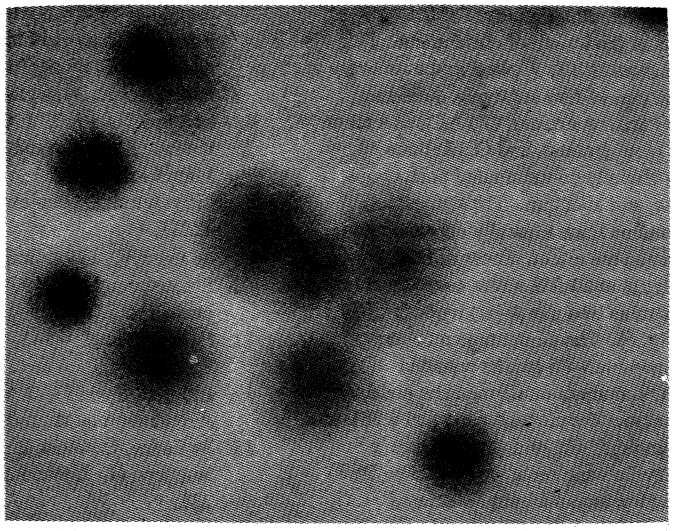


FIG. 6. Direct detection and isolation of YEP$^+$ strains as red pin point colon colonies on CR-BHO from naturally contaminated porcine tongue. (23)

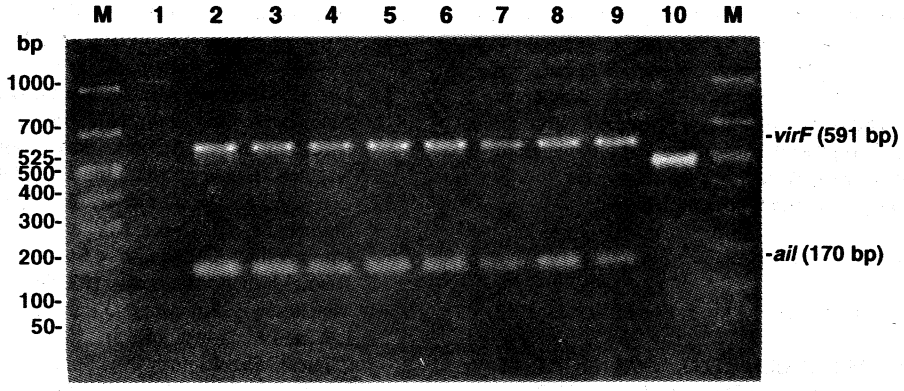


FIG. 7. Confirmation of CR$^+$ clones isolated from naturally contaminated porcine tongue as YEP$^+$ strains by multiplex PCR using chromosomal *ail* gene and *virF* gene from virulence plasmid. Lanes; M, 50 to 1000 bp ladder marker; 1, negative control with no template; 2-8, CR$^+$ colonies showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 9, positive control with purified DNA from YEP$^+$ strain showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and virulence plasmid respectively; 10, positive control for PCR assay with λ as DNA template. (23)

pork chops, and ground pork spiked with 10, 1 and 0.5 CFU/cm^2 (Table 3). Cheese and zucchini required an additional day of enrichment for detection of samples with an initial inoculum of 0.5 CFU/cm^2 (Table 3). YEP$^+$ strains could not be recovered from any of the samples at an initial contamination level of 0.1 CFU/cm^2 regardless of the length of enrichment. This technique has been successfully applied in the recovery of different YEP$^+$ strains of five serotypes including O:3 (five strains), O:8 (five strains), O:TACOMA (four strains), O:5, O:27 (four strains), and O:13 (three strains) from the artificially contaminated pork chops, ground pork, cheese, and zucchini. The successful isolation of YEP$^+$ strains from naturally contaminated porcine tongue verified the effectiveness of this method. Of 17 tongues analyzed, seven (~41%) were positive for YEP$^+$ strains (red pin point) by both CR binding and Lcr (Fig. 6). The percentage recovery of YEP$^+$ strain is comparable with results reported previously (14). PCR analysis confirmed the presence of a 170-bp product from the chromosome and a 591-bp product from the virulence plasmid (Fig. 7 lanes 2-8). All isolates from tongue were serotype O:3. The YEP$^+$ strains retained the virulence plasmid-associated phenotypic characteristics, and pathogenicity after isolation from foods by this procedure (12, 14).

In conclusion, the method described here has the following advantages: over the former method (i) requires only a single enrichment medium for a wide range of serotypes including a large number of different strains from a variety of foods, (ii) eliminates one day of enrichment and another for presumptive isolation, (iii) uses a single medium (CR-BHO) for direct detection and isolation, and (iv) preserves the virulence plasmid. This procedure is a practical alternative to many other recovery methods which require significantly more time for completion.

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